can be evaluated in vivo by using the acute-inflammation model in mice [12].

Acute inflammation is induced by the injection of zymosan into a dorsal air pouch created in a mouse and the subsequent accumulation of a large number of murine neutrophils into the air pouch [13,14]. Consequently, highly purified murine neutrophils with a sufficient capacity to migrate from other sites, such as the peripheral blood, into inflammatory sites can be isolated from mice by means of this inflammation model. Our objective was to investigate whether the zymosan-injection air pouch methodology can be used in immunodeficient mice with transplanted human HSC to elicit neutrophil migration and inflammation. This methodology gives us the opportunity to evaluate not only human neutrophil production but also critical functions of engrafted human neutrophils.

2. Materials and Methods

2.1. Animals

The experimental protocol was approved by the Committee of Animal Care and Experiments of the Research Institute of the International Medical Center of Japan (IMCJ) (protocol no. 17-Tg-7). NOG mice were purchased from the Central Institute of Experimental Animals (CIEA) (Kanagawa, Japan). All mice were kept under specific pathogen-free conditions at the animal laboratory of the Research Institute of IMCJ in accordance with CIEA guidelines.

2.2. Transplantation of Human CB CD34$^+$ Cells into Mice

Nine-week-old female NOG mice were sublethally irradiated with 2 Gy via an MBR1520-3 x-ray source (Hitachi Medical, Tokyo, Japan). After 24 hours, mice received intravenous transplants of 1.8×10^5 human CB CD34$^+$ cells (AllCells, Berkeley, CA, USA) or vehicle (saline). The purity and viability of the CB CD34$^+$ cells were greater than 95%.

2.3. Zymosan-Induced Air Pouch Inflammation

Six, 8, or 10 weeks after the transplantation of human CB CD34$^+$ cells, a subcutaneous air pouch was formed on the back of NOG mice, as has been described previously [13,14]. Five hundred microliters of zymosan solution (1 mg/mL in saline) was injected into the air pouch. Sixteen hours after zymosan injection, mice were decapitated under diethyl ether anesthesia, and the air pouch was washed with 1 mL of ice-cold phosphate-buffered saline (PBS) to obtain accumulated leukocytes.

2.4. Determination of Superoxide Release

Superoxide release stimulated by phorbol myristate acetate was assayed by the superoxide dismutase–inhibitable reduction of ferricytochrome c, which was monitored continuously in a Hitachi 556 double-wavelength spectrophotometer (Hitachi High-Technologies, Tokyo, Japan) equipped with a thermostatted cuvette holder (37°C), as described previously [11].

2.5. Preparation of Neutrophils from Human Peripheral Blood

Granulocytes and mononuclear cells were prepared from healthy adult donors as described previously [11] by using dextran (Nacalai Tesque, Kyoto, Japan) sedimentation, centrifugation with a separating solution (Lymphoprep; Axis-Shield, Oslo, Norway), and hypotonic lysis of the contaminating erythrocytes. Neutrophils constituted greater than 90% of the granulocyte fractions, and the remaining cells were eosinophils. Mononuclear cell fractions consisted of 20% monocytes and 80% lymphocytes. Both cell fractions were suspended in PBS containing 5% fetal calf serum (FCS).

2.6. Preparation of Murine Bone Marrow and Spleen Leukocytes

Bone marrow cells were harvested from the mice by flushing the femurs with ice-cold Hanks balanced salt solution (HBSS). Spleens were harvested from the mice and minced in ice-cold HBSS. The resulting cell suspensions were filtered through a nylon mesh, and contaminating erythrocytes were eliminated by hypotonic lysis. The cells were then washed once and suspended in PBS containing 5% FCS.

2.7. Determination of Cell Surface Antigens by Fluorescence-Activated Cell Sorting

All suspensions of single cells were stained with the appropriate antibodies and analyzed by fluorescence-activated cell sorting (FACS) with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cells were incubated with monoclonal antibodies for 30 minutes on ice in PBS containing 5% FCS. Nonspecific binding to cells bearing Fcγ receptors was blocked with a rat antimouse CD16/CD32 monoclonal antibody (BD Biosciences). The following monoclonal antibodies were used in this flow cytometric study: fluorescein isothiocyanate (FITC)-conjugated rat antimouse Ly-6G and Ly-6C antibody (Gr-1 antibody) (BD Biosciences), phycoerythrin (PE)-conjugated mouse antihuman CD45 (BD Biosciences), PE-conjugated mouse antihuman CD10 (BD Biosciences), FITC-conjugated mouse antihuman CD66b (Beckman Coulter, Miami, FL, USA), and each isotype as a control. Antimouse Gr-1 antibody, which reacts selectively with murine neutrophils, does not cross-react with human hematopoietic cells, including neutrophils, and antihuman CD45, CD10, and CD66b antibodies, which react selectively with human neutrophils, do not cross-react with murine neutrophils.

2.8. Immunocytochemical Study

Cells collected from air pouches were washed with PBS and fixed on glass slides by means of a Cytospin apparatus (Cytospin 2; Shandon, Pittsburgh, PA, USA). After further fixation with acetone/methanol solution (1:3), immunostaining was performed as described previously [15] by using FITC-conjugated antihuman CD16b monoclonal antibody (Beckman Coulter) or antihuman CD45 monoclonal

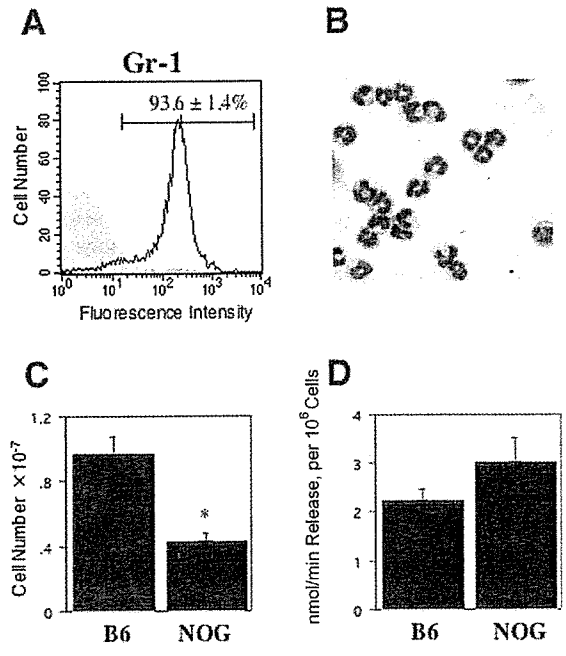

Figure 1. Air pouch inflammatory model in nonobese diabetic/severe combined immunodeficiency/γ chain^{null} (NOG) mice. Zymosan suspended in saline (0.5 mg/mouse) was injected into the air pouch of NOG mice to induce inflammation. The pouch was washed with phosphate-buffered saline 16 hours after zymosan injection to obtain accumulated leukocytes. A, Fluorescence-activated cell-sorting analysis of the expression of the granulocyte-specific antigen Gr-1 was carried out with leukocytes from the air pouch of NOG mice (n = 3). B, Morphology of the leukocytes in the air pouch of NOG mice. After fixation to glass slides, cells were stained with Wright-Giemsa solution and examined by light microscopy. C, The number of leukocytes in the air pouch was determined in B6 and NOG mice. D, The respiratory burst activities of granulocytes in the air pouch in B6 and NOG mice. Superoxide (O_2^-) release stimulated by 100 ng/mL phorbol myristate acetate was determined by the reduction of cytochrome *c* and is expressed as nmol/minute per 10^6 cells. Data for (C) and (D) are expressed as the mean ± SE (n = 6), and statistical analysis was performed by means of an unpaired Student *t* test. *$P < .05$, NOG versus B6 mice.

antibody (BD Biosciences). For the control, an isotype antibody reaction was performed by using FITC-conjugated immunoglobulin M (IgM) κ (ICN Biomedicals, Aurora, OH, USA) and IgG1 κ (BD Biosciences) for the anti-CD16b and anti-CD45 antibody reactions, respectively. The second antibody reaction was performed by using Alexa Fluor 488 goat antimouse IgM (Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 488 goat antimouse IgG (Invitrogen) for the anti-CD16b and anti-CD45 antibody reactions, respectively.

2.9. Morphologic Observation

Cells were fixed on glass slides with a Cytospin 2 apparatus, stained with Wright-Giemsa solution (Muto Pure Chemical, Tokyo, Japan), and then observed with a light microscope (Olympus Optical, Tokyo, Japan).

2.10. Statistical Analysis

Statistical analysis was performed by means of the unpaired Student *t* test and the StatView software package (version 5.0; SAS Institute/Abacus Concepts, Berkeley, CA, USA).

3. Results

3.1. Zymosan-Induced Air Pouch Inflammation in NOG Mice

The leukocytes that accumulate by zymosan-induced air pouch inflammation in normal mice are known to be predominantly neutrophils, along with small numbers of monocytes and lymphocytes (data for normal B6 mice are not shown) [13,14]. In the present study, we first investigated whether zymosan-induced accumulation of mature murine neutrophils into the air pouch also occurs in NOG mice.

As has been observed with this inflammatory model in normal mice, the leukocytes that accumulated in the air pouch after zymosan injection in NOG mice were predominantly neutrophils. This result was established by detecting the expression of a specific surface antigen of mouse neutrophils, Gr-1, as well as the typical morphologic characteristics obtained via Wright-Giemsa staining (Figures 1A and 1B). Both the FACS analysis of the Gr-1 antigen and the morphologic evaluation indicated that greater than 90% of the accumulated leukocytes were neutrophils. In the NOG mice lacking lymphocytes, the remaining leukocytes (<10%) were monocytes (data not shown).

These results indicate that the migration of neutrophils toward inflammatory sites occurs almost normally in NOG mice, suggesting that the neutrophils of NOG mice have a normal chemotactic function. The number of leukocytes in the air pouch of NOG mice, however, was significantly lower ($P < .05$) than that of B6 mice (Figure 1C). On the other hand, the respiratory burst activity, another important function of neutrophils, was normal, because agonist-induced superoxide release from the leukocytes in the air pouch of NOG mice was equivalent to that of B6 mice (Figure 1D).

3.2. Engraftment of Human CB CD34⁺ Cells in NOG Mice

We next transplanted human CB CD34⁺ cells into recipient NOG mice. After being sublethally irradiated, NOG mice received intravenous transplants of human CB CD34⁺ cells. Six weeks after transplantation, leukocytes in the bone marrow and spleen were analyzed for the expression of the human panleukocyte marker, CD45 (Figure 2). In these hematopoietic organs, approximately 90% of the cells expressed human CD45 antigen, both at 6 weeks and at 8 weeks after the transplantation of human CB CD34⁺ cells (Figure 2). These findings indicate the highly effective engraftment of human hematopoietic cells in this immunodeficient mouse, and the efficiency of engraftment obtained in

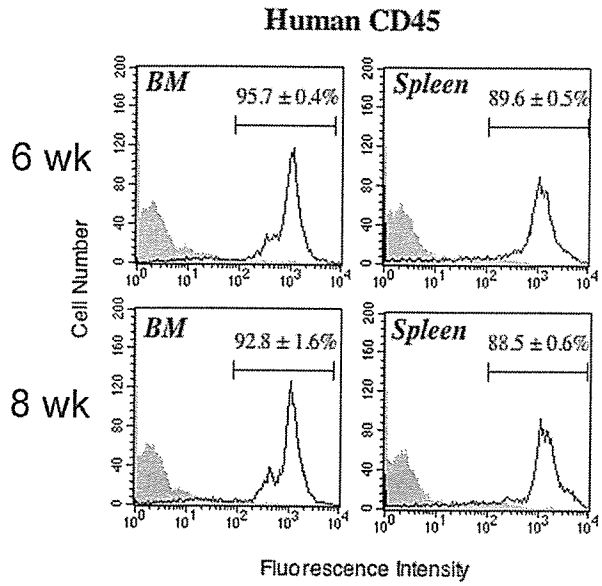

Figure 2. Engraftment of human cord blood CD34⁺ cells in nonobese diabetic/severe combined immunodeficiency/γ chain^{null} (NOG) mice. CD34⁺ cells from human umbilical cord blood (1.8×10^5 cells/mouse) were transplanted into NOG mice intravenously. Bone marrow (BM) and spleen leukocytes were obtained at 6 and 8 weeks after transplantation as described in "Materials and Methods." Cell surface expression of human CD45 was determined by fluorescence-activated cell-sorting analysis. Data are expressed as the mean ± SE (n = 3).

this study was equivalent to or better than the results described in previous reports [3,4].

We then evaluated whether CD45⁺ human hematopoietic cells appear in the air pouch of NOG mice that had received transplants of human CB CD34⁺ cells. As is shown in Figure 3 (upper panel), more than 90% of the leukocytes in the air pouch of NOG mice infused with vehicle alone were Gr-1⁺ murine neutrophils, and there were no human CD45⁺ leukocytes. In contrast, a significant level (approximately 10%) of human CD45⁺ cells in the air pouch was present in NOG mice that had received transplants of human CB CD34⁺ cells, and there was a concomitant decrease in the percentage of Gr-1⁺ murine neutrophils. We observed these findings at both 6 weeks and 8 weeks after transplantation of human CB CD34⁺ cells, although the human CD45⁺ cells in the air pouch had decreased at 8 weeks for an unknown reason.

3.3. Identification of Human Neutrophils Accumulated by Zymosan-Induced Air Pouch Inflammation in Mice with Transplanted Human CB CD34⁺ Cells

Because most of the leukocytes in zymosan-induced air pouch inflammation were neutrophils, the human CD45⁺ leukocytes in Figure 3 were considered human neutrophils that had differentiated in vivo in the NOG mice. To further confirm this hypothesis, we performed FACS analysis with monoclonal antibodies that specifically recognize mature human neutrophils. We selected 2 neutrophil-specific cell surface molecules, CD10 and CD66b. CD10, well known as a common acute lymphoblastic leukemia antigen [16], has been reported to be expressed in mature neutrophils [17-19], and CD66b is a specific cell surface antigen of human granulocytes [20].

Before the transplantation experiments with NOG mice, we performed several experiments with normal human neutrophils and mononuclear leukocytes to establish experimental conditions for the 2-color flow cytometric analysis of CD10 and CD66b. As is shown in Figure 4 (upper panel), we successfully performed 2-color analysis with a granulocyte fraction isolated from a healthy donor. The granulocyte fraction contained 96.5% CD66b⁺ granulocytes, which consisted of 86.3% CD10⁺ neutrophils and 10.2% other CD10⁻ granulocytes, probably eosinophils. This granulocyte fraction contained more than 98% CD45⁺ cells (data not shown). In contrast, the cells in mononuclear cell fractions were negative for both CD66b and CD10.

Using this analytical condition, we then performed a 2-color flow cytometric analysis of the leukocytes that had

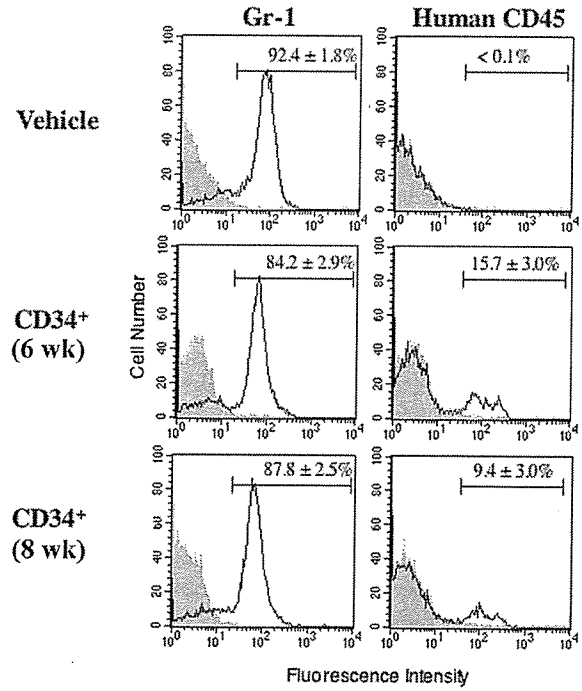

Figure 3. Identification of human leukocytes in the air pouch of nonobese diabetic/severe combined immunodeficiency/γ chain^{null} (NOG) mice. CD34⁺ cells from human umbilical cord blood (1.8×10^5 cells/mouse) (CD34⁺) or saline (vehicle) was transplanted into NOG mice intravenously. Zymosan was suspended in saline (0.5 mg/mouse) and injected into the air pouch to induce inflammation by 6 or 8 weeks after transplantation. The pouch was washed with phosphate-buffered saline 16 hours after zymosan injection to obtain accumulated leukocytes. Cell surface expression of murine Gr-1 and human CD45 was determined by fluorescence-activated cell-sorting analysis. Data are expressed as the mean ± SE (n = 3).

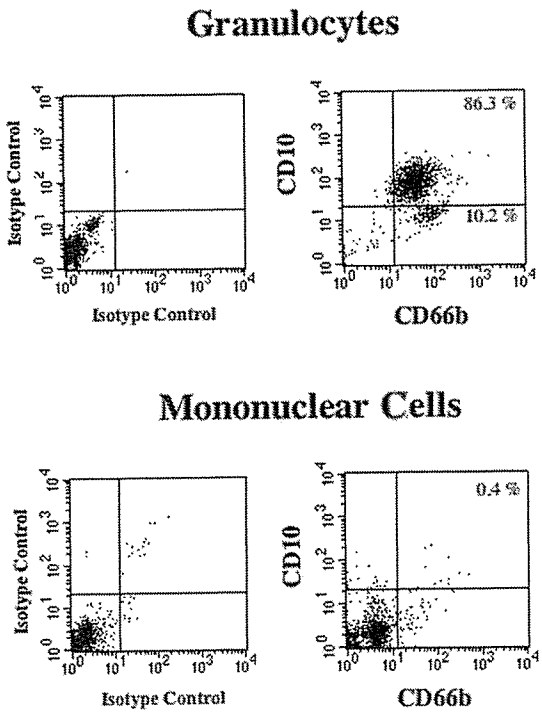

Figure 4. Two-color fluorescence-activated cell-sorting (FACS) analysis of CD10 and CD66b in human peripheral blood leukocytes. Human granulocytes and mononuclear cells were isolated as described in "Materials and Methods." Two-color FACS analysis of the cell surface expression of human CD10 and CD66b was performed with each isotype antibody as a control.

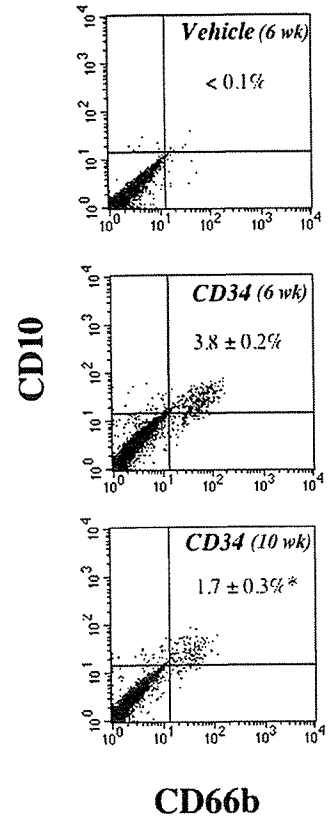

Figure 5. Two-color fluorescence-activated cell-sorting (FACS) analysis of CD10 and CD66b in leukocytes in the zymosan-induced air pouch in nonobese diabetic/severe combined immunodeficiency/γ chain^{null} (NOG) mice with transplanted human umbilical cord blood (CB) CD34$^+$ cells. Human CB CD34$^+$ cells (1.8×10^5 cells/mouse) were transplanted into NOG mice intravenously. Zymosan suspended in saline (0.5 mg/mouse) was injected into the air pouch to induce inflammation at 6 or 10 weeks after transplantation (middle and lower panels). Sixteen hours after zymosan injection, the pouch was washed with phosphate-buffered saline to obtain accumulated leukocytes. Cell surface expression of human CD10 and CD66b was determined by FACS analysis. As a negative control, leukocytes that had accumulated in the air pouch in NOG mice without CD34$^+$ cell transplantation were analyzed, and the results are shown in the top panel. Data are expressed as the mean ± SE (n = 3). Statistical analysis was performed by means of an unpaired Student *t* test. $^*P < .05$, 10 weeks versus 6 weeks.

accumulated in the air pouch after zymosan injection into mice with transplanted human CB CD34$^+$ cells. As is shown in Figure 5, double-positive (both CD10$^+$ and CD66b$^+$) human neutrophils were detected in the air pouch at both 6 weeks and 10 weeks after the transplantation of human CB CD34$^+$ cells. It is interesting that the double-positive human neutrophils in the air pouch had decreased by 10 weeks, compared with the numbers at 6 weeks. These data for double-positive human neutrophils in the air pouch almost corresponded to the data estimated from the numbers of human CD45$^+$ leukocytes in the air pouch (data not shown).

Finally, we used immunocytochemical staining to confirm that human neutrophils actually existed in the air pouch following zymosan injection into mice with transplanted human CB CD34$^+$ cells. As is shown in Figure 6, we were able to detect human CD45$^+$ cells with a neutrophil morphology in the leukocytes that had accumulated in the air pouch of mice with transplanted human CB CD34$^+$ cells. In contrast, human CD45$^+$ cells were not observed in the leukocytes that had accumulated in the air pouch of mice that had not undergone transplantation (data not shown). The presence of human neutrophils in the air pouch of mice with transplanted human CB CD34$^+$ cells was also confirmed with antihuman neutrophil-specific CD16b antibody (Figure 7).

Thus, using an in vivo inflammatory model, we have shown the functional engraftment of human neutrophils.

4. Discussion

Mature human neutrophils derived from human hematopoietic progenitor cells have not been identified in the peripheral blood of immunodeficient mice with transplanted human HSC, although HSC are considered to dif-

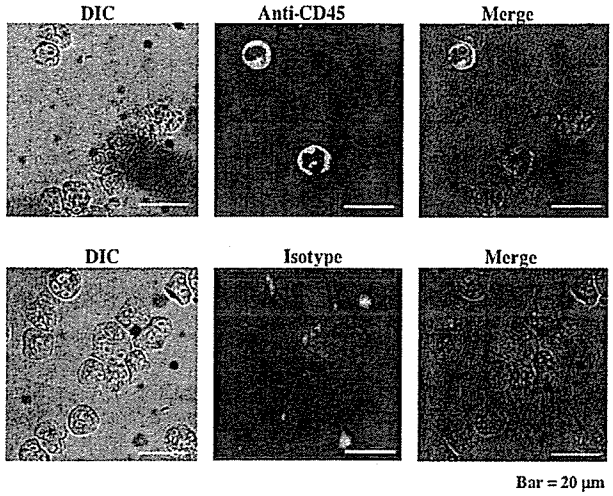

Figure 6. Immunocytochemical analysis of CD45 in leukocytes in the zymosan-induced air pouch in nonobese diabetic/severe combined immunodeficiency/γ chain^{null} (NOG) mice with transplanted CD34$^+$ cells from human umbilical cord blood (CB). Human CB CD34$^+$ cells (1.8×10^5 cells/mouse) were transplanted into NOG mice intravenously. Zymosan suspended in saline (0.5 mg/mouse) was injected into the air pouch to induce inflammation at 6 weeks after transplantation. The pouch was washed with phosphate-buffered saline 16 hours after zymosan injection to obtain accumulated leukocytes. Immunocytochemical analysis of human CD45 expression (top) was performed with isotype antibody as a control (bottom), and the staining pattern was observed with an inverted microscope. The left panels are difference interference contrast (DIC) images, the middle panels are the corresponding images obtained by fluorescence microscopy, and the right panels are combined images of the corresponding left and middle panels.

ferentiate into all hematopoietic cell lineages, including neutrophils. In the present study, we established a novel system to detect human neutrophils in NOG mice with transplanted human CB CD34$^+$ cells by using a model of experimentally induced inflammation and a 2-color FACS analysis that used 2 monoclonal antibodies specific for mature human neutrophils and/or granulocytes (Figure 5). In addition, the results obtained with our system, which selectively detects neutrophils that have migrated into the site of inflammation, indicated that the functional human neutrophils had developed from human hematopoietic progenitor cells in vivo.

Analysis of the cell surface antigens of leukocytes by FACS has been widely performed in many immunologic and hematologic studies because morphologic identification has not been capable of distinguishing lymphocyte subpopulations. In contrast, studies for specific cell surface markers of human neutrophils have been extremely limited, because human neutrophils are easily recognized by their morphology and there are no distinct subpopulations with the same morphology. However, to distinguish precisely between human and mouse neutrophils in chimeric mice with transplanted human hematopoietic progenitor cells requires determining specific cell surface antigens of human neutrophils. In the murine system, Gr-1 is a well-known granulocyte-specific cell surface marker and is known as a common epitope of Ly-6 subtypes in mice [21]. Stroncek et al reported that CD177, known as human neutrophil-specific antigen NB1, was a member of the Ly-6 gene superfamily and that it might be a human counterpart of Gr-1 [22]. However, the level of CD177 expression in human neutrophils is approximately 50% (unpublished data), suggesting that CD177 is not suitable for the complete identification of human neutrophils. In contrast, the present study clearly revealed that human neutrophils could be identified by a 2-color FACS analysis of 2 cell surface antigens, CD10 and CD66b, which are specific markers for granulocytes and/or neutrophils (Figure 4).

We found that neutrophil accumulation during zymosan-induced acute inflammation was reduced in NOG mice, compared with B6 mice (Figure 1C). Zymosan is known to activate the alternative pathway of complement and C5a formation [12], and inflammatory mediators, such as arachidonate metabolites, that are released from resident macrophages upon zymosan stimulation cause neutrophils to accumulate at the site of inflammation [23]. NOG mice have multiple immunologic defects in innate immunity, including a lack of macrophage function, complement-dependent hemolytic activity, and natural killer cell activity [3]. Therefore, the reduction of macrophage function and complement-

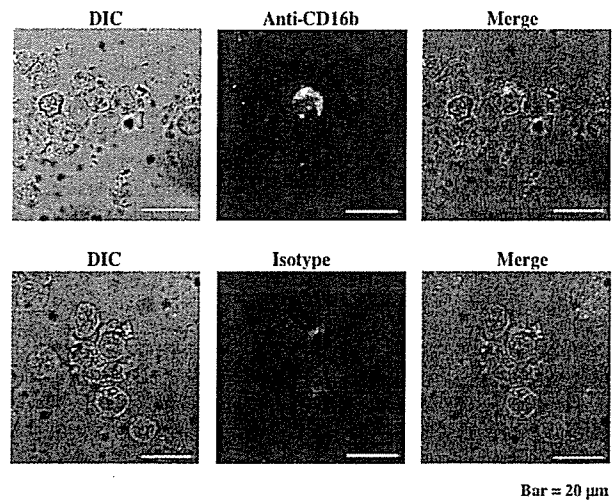

Figure 7. Immunocytochemical analysis of CD16b in leukocytes in the zymosan-induced air pouch in nonobese diabetic/severe combined immunodeficiency/γ chain^{null} (NOG) mice with transplanted CD34$^+$ cells from human umbilical cord blood (CB). Human CB CD34$^+$ cells (1.8×10^5 cells/mouse) were transplanted into NOG mice intravenously. Zymosan suspended in saline (0.5 mg/mouse) was injected into the air pouch to induce inflammation at 6 weeks after transplantation. The pouch was washed with phosphate-buffered saline 16 hours after zymosan injection to obtain accumulated leukocytes. Immunocytochemical analysis of human CD16b expression (top) was performed with isotype antibody as a control (bottom), and the staining pattern was observed with an inverted microscope. The left panels are difference interference contrast (DIC) images, the middle panels are the corresponding images obtained by fluorescence microscopy, and the right panels are combined images of the corresponding left and middle panels.

dependent hemolytic activity may account for the reduction of neutrophil accumulation during zymosan-induced acute inflammation in NOG mice. On the other hand, superoxide-producing capacity, another important function of neutrophils, was normal in NOG mice (Figure 1D). Therefore, the development and function of neutrophils are considered normal to some degree in NOG mice.

Previous studies have shown that engraftment of human $CD45^+$ cells in NOG mice is lymphocyte predominant and that engraftment increases gradually for 4 to 12 weeks following the transplantation of human CB $CD34^+$ cells [3,5,6]. On the other hand, we found that the engraftment of human neutrophils as estimated by our in vivo air pouch inflammatory model declined from 6 to 10 weeks after the transplantation of human $CD34^+$ cells (Figures 3 and 5). Although the exact reason for these different observations remains unclear, there are 2 possibilities: (1) extremely different life cycle and production kinetics for neutrophils and lymphocytes, or (2) the relatively normal development of murine neutrophils versus an almost complete lack of murine lymphocytes in NOG mice.

Investigators have recently concluded that human embryonic stem cells constitute a valuable resource for regenerative medicine because of their high capacity to differentiate into a broad range of cell types [24]. Consequently, many researchers have been vigorously studying such cells to establish culture conditions and techniques for the in vitro differentiation of human embryonic stem cells into hematopoietic stem or progenitor cells [25,26]. For clinical applications, however, both the differentiation and therapeutic potential of such cells should be investigated and evaluated in vivo, although performing such in vivo studies is not allowed in human beings. Therefore, our system may be useful as an in vivo system to evaluate the hematopoietic activities, particularly granulopoietic, of human hematopoietic progenitor cells derived from human embryonic stem cells.

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